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Studies on the Separation of α-Amylase from Malt Extract

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In the previous paper, it was indicated that the detergents of all types, with the exception of non-ionic one, inhibited strongly the amylases under certain conditions, and α-Amylase, in general, was more stable than β-amylase against the detergent inhibition. New separation methods of malt α-amylase from accompanying β-amylase with the usage of cationic detergents are described in this paper.

INTRODUCTION

The constitution of the amylolytic enzyme system in malt has been studied extensively with regard to the separation, characterization and accurate determination of the components.

The methods of Waldschmidt-Leitz, Reichel and Purr[1] (1932) for the selective adsorption of β-amylase on alumina at pH 3.8, or the adsorption of α-amylase on mature starch grains according to Holmbergh[2] (1933) are well known, but their methods are not suitable for the quantitative separation of the α-amylase from the β-amylase. The Ohlsson[3,4] technipue can be used to destroy the β-amylase in the presence of α-amylase by heating the neutral green malt extract at 70° for 15 minutes (1926, 1930). Several workers, including Ohlsson[3] (1926) however, have found that at least 15-16% of the α-amylase is destroyed by this treatment.

In the present experiment, it was pointed out that the method of Ohlsson and its modification (Kneen's method[5]) using Ca-salt were not satisfactory for the complete separation of the α-amylase from the β-amylase. It was also found that in the Osvan treatment (corresponding to the cationic detergent) the α-amylase was stable whereas the β-amylase was much labile. The new method for the complete separation of the α-amylase from the malt extract was established experimentally.

1. INVESTIGATION ON THE METHOD OF OHLSSON

1. Separation of α-Amylase by the Method of Ohlsson. Malt extract (1 : 5) and pure amylase were treated by Ohlsson's method, i. e., by heating at 70° for 15 minutes, on various concentrations. The effect of Ca-ion on the treatment was examined (Fig 1). Activity remained was determined by the method as described previously.

Fig. 1 indicates that Ohlsson's and Kneen's methods are not suitable to separate the α-amylase from the accompanied β-amylase without any loss,

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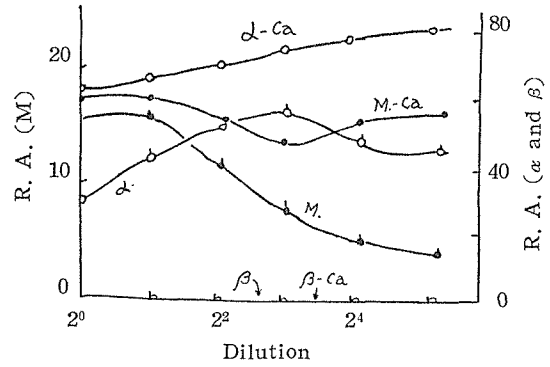

Fig. 1. The effect of heating at 70°C on the separation of α-amylase.
R. A., Remaining Activity ; α-Ca, pure α-amylase containing $CaCl_2$; M-Ca, malt extract containing $CaCl_2$; α, pure α-amylase ; M, malt extract ; β-Ca, pure β-amylase containing $CaCl_2$, β, pure β-amylase.

although these methods have generally been used.

2. Separation of β-Amylase by the Method of Ohlsson. Barley extract (1:5), green malt extract (1 : 5) and pure amylase were treated respectively by various concentrations. The activities of the samples treated were determined as described previously.

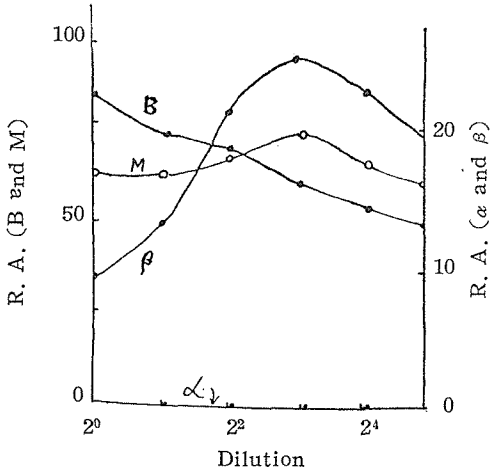

Fig. 2. The effect of Ohlsson's treatment on the separation of β-amylase.
R. A., Remaining activity; B, barley extract ; M, malt extract; β, β-amylase ; α , α-amylase.

This treatment showed an incomplete separation of the β-amylase, and the activity remained was high at the high concentration of the barley extract, whereas it was low in the low concentration. This fact shows that the α-amylase is accompanied in the case of the high concentration of the barley extract, but in the low concentration β-amylase is destroyed to some extent. In the case of the malt extract, the remaining activity was almost constant, but its value was lower than the theoretical one.

Thus it is concluded that Ohlsson's method is unsuitable for the separation of one of the amylases, and, especially, its application on the pure amylase, since remarkable destruction of the both amylases are inevitable. Thus Ohlsson's method must be improved.

II. OSVAN (A CATIONIC DETERGENT) TREATMENT ON THE MALT AMYLASES

1. The Effect of Temperature on the Amylase Inhibition by Osvan. It was shown previously that the inhibitory effect of detergents on the amylase increased with increasing temperature. In the present experiment, the pure α-and β-amylase were kept in contact with Osvan for 30 min. at 20°C. The concentration of Osvan was 0.05% in final. Then they were heated at the temperatures between 40° and 70°C for 5 min. Activity remained was determined at pH 4.7 as described previously.

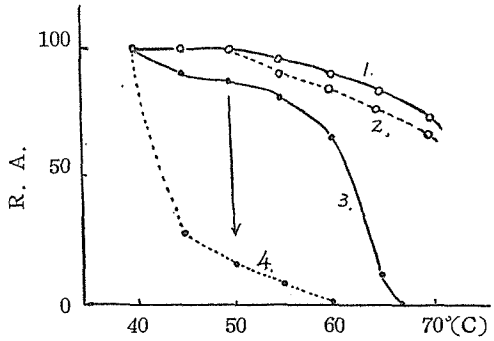

Fig. 3 The effect of temperature on th Osvan treatment.
R. A,. Remaining activity ; 1, α-amylase untreated ; 2, α-amylase treated with Osvan ; 3, β-amylasee untreated ; 4, β-amylase treated with Osvan.

As shown in Fig. 3, the thermostability of the β-amylase which was in contact with Osvan, decreased remarkably, whereas the α-amylase was thermostable. For instance, at 50°C, the activity of the β-amylase was reduced to about 20 % of its initial value when treated with Osvan, and to about 90 % when not treated. Treated under the same condition, however, the α-amylase thermostable and its activity remained almost constant. The difference in the thermnstability btween the α-and β-amylase, could be used for the separation of the former without any loss from the latter.

2. The Effect of the Concentration of Enzyme on the Thermostability. In general, the thermostability of enzyme diminishes according to the reduction with the decrease of its concentration. In the case of the Osvan-treatment, the relationshop between the concentration of amylase and its thermostability is shown in Fig. 4.

The Osvan inhibition on the β-amylase increased in proportion to the concentration of Osvan, and inversely to that of the β-amylase. The effect to Caion on the Osvan treatment is protective for α-amylase and inhibitory for β-

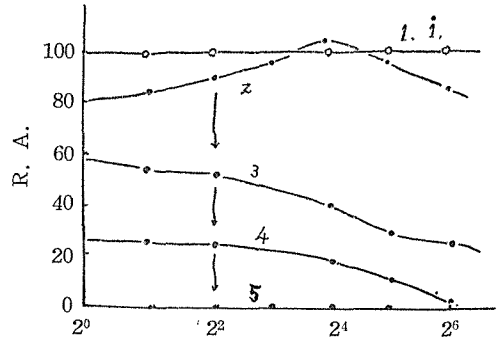

Fig. 4. The effect of enzyme concentration on the Osvan treatment at 50° for 10 min.

1. α-amylase ; i, α-amylase (Osvan 0.04 %, $CaCl_2$ 0.1 M)* 2, β-amylase ; 3, β-amylase (Osvan 0.025%)* 4, β-amylase (Osvan 0.04%)* 5, β-amylase (Osvan 0.04 %, $CaCl_2$ 0.1M)*

*Conditions when the treatments were carried out.

amylase. From the results described above, a new technique which is more favorable than Ohlsson's and Kneen's techniques, was developed. The new technique consists in treating a sample at 50° for 10 minutes with 0.1% Osvan, 0.1 M $CaCl_2$ in final concentration was used to separate the α-amylas from the accompanying β-amylase. Pure α-and β-amylase, and barley extract containing no α-amylase were mixed in the proportion as listed in Table 1. After these samples were treated with the new technique described above, their remaining activities were determined at pH 4.7.

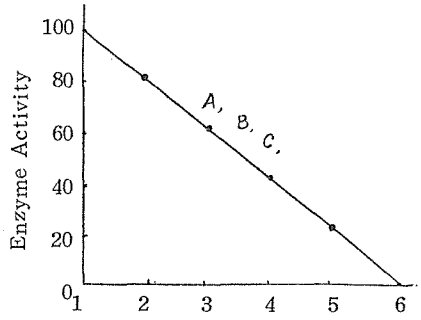

Fig. 5. The effect of Osvan treatment on amylase mixtures.

Table 1. Proportion of α-, β-amylase and barley extract in the mixtures.

Materials	No. of sample 1	2	3	4	5	6	Symbols in the Fig. 5.
α-amylase	10	8	6	4	2	0	A
β-amylase	0	2	4	6	8	10	
α-amylase	10	8	6	4	2	0	B
Barley extract	0	2	4	6	8	10	
α-amylase	10	8	6	4	2	0	C
Distilled water	0	2	4	6	8	10	

In Table 1, it is interesting to see that the β-amylase is inhibited completely and the α-amylase is left unchanged without any loss irresepective of the proportions of the components in the mixtures. This results indicate that the new treatment is more suitable for the separation of the α-amylase from the mixture containing the β-amylase.

Besides Osvan, other cationic detergents were examined for the ability to separate the α-amylase. To investigate inhibition of β-amylase, the β-amylase

was mixed with cationic detergent of several types, and kept at 50° for 10 min. The effect of the addition of Ca-ion was also examined. The results are shown in Table 2.

Table 2. Comparison of the effect of cationic detergents on the inhibition of β-amylase. (Remaining activities are expressed in the percentage of the inhitial values.)

Detergents*	Concn. of detergent in final (%)							
					0.1**		0.05	
	0.2	0.1	0.05	0.025	$CaCl_2$ 0.1*M*	$CaCl_2$ 0.01*M*	$CaCl_2$ 0.1*M*	$CaCl_2$ 0.01*M*
HD	3.1	8.3	21.0	53.0	0	3.1	1.6	15.0
M_2-100	0	0	1.0	7.3	0	0	0	0
Hyamin	0	0	10.0	26.6	0	0	1.6	20.0
Osvan	0	0	2.3	33.2	0	0	2.0	2.6

*HD : Lauryl pyridinium chloride, M_2—100 : Dimethyl myristyl benzyl ammonium chloride, Hyamine : Benzethonium chloride, Osvan : Alkyl dimethyl ammonium chloride.

**The α-amylase is left without any loss under the condition.

The inhibitory power was different according to the constitution of the detergent. The inhibitory effect on each treatment was enhanced by the addition of $CaCl_2$. In the treatment with 0.1% cationic detergents and 0.1*M* $CaCl_2$ in final, pure β-amylase was destroyed completely whereas α-amylase was stable without any loss. It appears that every cationic detergents have the same behaviors as Osvan against α-amylase and β-amylase.

3. The Effect of Impurities on Amylases. The new technique described above was investigated, furthermore, with respect to the effect of several substance introduced as impurities into the test solutions.

(i) The effect of the concentration of malt extract : The malt extracts (1 : 5. originally) of the various concentration were treated with 0.1% Osvan and 0.1*M* $CaCl_2$ in final, at 50° and 55° respectively for 10 min. The results shown in Table 3 suggested that, when the temperature of the treatment was 50°C, the β-amylase remained to some extent for the high concentration of malt extract, while, when the temperature was raised to 55°C, the β-amylase was removed completely.

Table 3 The effect of the concentration of malt extract on the Osvan treatment. (Remaining activities are expressed in the precentage of the initial values.)

Treatment at	Dilution				
	2^0	2^1	2^2	2^4	2^6
50°, 10min.	28.3	26.5	22.3	20.6	17.8
55°, 10min.	17.8	17.8	17.8	17.8	17.8

(ii) The effect of proteins : The new treatment was given to the β-amylase containing 0.5% proteins in final, at 50° and 55° for 10 min.

Table 4. Comparison of the effect of proteins on the inhibition of the β-amylase in the new treatment. (Remaining activity is expressed in the percentage of the initial value.)

Temp.	Control (no-added)	Papain	Gelatin	Casein	Peptone
50°	0	2.8	0.3	61.9	0.80
55°	0	0	0	0	0

Marked protection against the inhibition in the Osvan treatment was observed in the case of casein at 50°C. This fact would be due to the combination of Osvan with the casein, and, subsequently, to the reduction of the free Osvan. In the treatment at 55°, however, the complete inhibition of β-amylase was observed as seen in Table 4.

(iii) The effect of starch and its components : It has been well known that starch resembles protein with respect to the behavior to combine with Osvan. The protective effect of starch and its components was examined under the same condition as in the case of protein. β-capmylase was mixed with raw starch, amylose and amylopectin, respectively, which were all prepared from commercial potato starch, and were subjected to the treatment at 50° and 55°. A little protection by starch and its components was found in the treatment at 50°, but no protection at 55°.

Table 5. The protective effect of starch and its components on the inhibition of β-amylase in the new treatment. (The remaining activity is shown in the table.)

Temp.	Soluble Starch 1%	Soluble Starch 2%	Raw Starch 2%	Amylose 0.5%	Amylopectin 0.5%
50°	3.5	11.9	0	3.9	2.4
55°	0	0	—	0	0

Amount of substances added were expressed in the percentage of the final concentratin of the solution treated. The investigation on the effect of a series of Osvan and other sbstances on the amylase has led to the development of a new quantitative method for the separation of the α-amylase from the accompanying β-amylase. The method consists in treating the sample containing amylases, malt and barley extract at 50° for 10 min, with 0.1% Osvan (a cationic detergent) and 0.1*M* $CaCl_2$ in final concentration. [For the special case in which the malt extract is high in concentration or the samples contain some proteins or starch and its components, the temperature in the treatment was raised to 55°.

III. OSVAN-ALKALINE TREATMENT ON MALT AMYLASES

The effect of pH on the detergent treatment was described in the previous report. It was found that the inhibition of amylase by Osvan was accelerated strongly on the alkaline side. In the presence of Ca-ion, however, the α-amylase was protected but the β-amylase was not. Thus a new treatment was developed for the separation of the α-amylase from the β-amylase.

1. The Effect of Ca-ion on the Osvan-alkaline Treatment. Pure amylase was mixed with Osvan (0.1% in final), and kept at 30° for 20 min. The pH of the solution was adjusted with N/20 NaOH between 7.0 and 9.0. Fig. 6 indicates the effect of the addition of $CaCl_2$. $CaCl_2$ was added to the amylase mixtures before the treatment, so that its concentration became 0.1*M* in final.

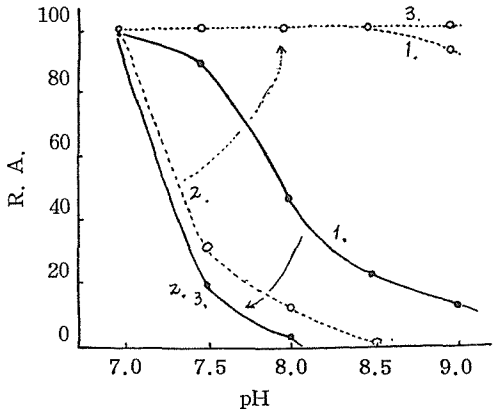

Fig. 6. The effect of $CaCl_2$ (0.1*M* in final) on the Osvan-alkaline treatment.
·····○·····, α-Amylase ; ——●——, β-amylase.
1, untreated; 2, treated (in the absence of $CaCl_2$) ; 3, treated (in the presence of $CaCl_2$).

In the absence of $CaCl_2$, both amylases were inhibited strongly in the Osvan treatment at pH 5.5, while in the presence of $CaCl_2$, the α-amylase showed high stability. Considering the results shown in Fig. 6, the author has developed a new method named an Osvan-alkaline method, in which the sample was mixed with Osvan 0.1% in final and $CaCl_2$ 0.1*M* in final and was heated at 30° for 20° min. By this method a complete separation of the α-amylase from the β-amylase was made possible.

***2.* The Effect of the Impurities on the Amylase.** The effects of the concentration of malt and barley extract, of proteins and starch mixed with the amylase solution as impurities was investigated as follows.

(i) The effect of the concentration of barley and malt extract : The Osvan-alkaline method described above was carried out with the barley and malt extract of various concentrations. The pH of sample was adjusted with buffer solution (0.2*M* phosphate-NaOH) to 9.0. The activity remained was determined after the pH of the sample was adjusted to 4.7 with 0.2 *M* acetate buffer.

Table 6. The effect of the concentration of barley and malt on the Osvan-alkaline treatment. (The remaining activity is expressed in the percentage of the initial value.)

Concentration of extract	Barley extract	Malt extract
1 : 5	7.08	20.69
1 : 10	0	20.69
1 : 20	0	20.69

As can be seen in Table 6, the barley extract still maintained its activity when its concentration was high (1 : 5) while it lost its activity completely when its concentration was low (1 : 10, 1 : 20). For the malt extract, however, the activity remained constant. This would indicate that the β-amylase in the malt extract is lost and the α-amylase is left unchanged Therefore, it appears that the Osvan-alkaline treatment is suitable for the separation of the α-amylase in the barley and malt extracts if the concentration of the extracts is regulated to be low.

(ii) The effect of proteins : Different kinds of proteins as interfering substances against Osvan-inhibition of amylase were investigated. Pure β-amylase solutions which were mixed with proteins were treated with Osvan at pH 9.0 as described above. After the treatment the remaining activities were determined (Table 7.)

Table. 7. Comparison of the effects of proteins on the Osvan inhibition of the β-amylase.

Final conc.*	Proteins				
	Nil	Papain	Gelatin	Casein	Peptone
0.25 %	0	1.48	0	8.89	0
0.50	0	2.44	0	65.90	0

* Final concentration of protein in the mixture solution.

As can be seen in Table 7, there is marked protection by casein. The same result was obtained previously in the case of the Osvan treatment at 50°.

In general, a marked precipitation is osberved when casein is mixed with Osvan solution, where the inhibitory effect abolish.

(iii) The effects of starch and its components : Raw starch, soluble starch, amylose and amylopectin, which were all prepared from commercial potato starch, were added respectively into pure β-amylase, and then, was given the Osvan alkaline technique at pH 8.85-8.90. The activities remained were listed in Table 8.

Table 8. Comparison of the effects of starch and its components on the Osvan inhibition of the β-amylase.

Substances added*	Nil	Soluble starch 1%	Amylose 0.25%	Amylopectin 0.25%	Raw starch 1.0%
Activity remained (%)	0	3.7	4.4	2.2	0

*Percentages of substance added were expressed in the final concentration of the solution treated.

The protective effects were observed for almost all of the starches tested. This result is in accordance with that obtained by the Osvan treatment at 50° described previously. The fact that the protection by the amylose is stronger than the amylopectin seems to suggest that the Osvan combines better with amylose than amylopectin. Contrary to the inhibitory effect of the β-amylase in the Osvan alkaline treatment, the stability of the α-amylase treated under the same condition was found.

The investigations on the effects of a series of amylase inhibition gave an optimum condition for the separation of the α-amylase from the β-amylase, i.e., the sample should be mixed with 0.1% Osvan and 0.1 *M* $CaCl_2$, in final concentration respectively at pH 9.0 and heated at 30°C for 20 min. This method, however, can not be used in this special cases in which the β-amylase contains casein or barley extract in high concentration.

IV. THE EFFECT OF THE OSVAN TREATMENT ON VARIOUS AMYLASES

The two different Osvan techniques, which were used for the determination of the malt α-and β-amylase were investigated in respect to their inhibitory effects on various amylases. Amylases tested were obtained from the following sources.

Malt amylases : β-and α-amylase were prepared from malt extract in higher purity by the methods of Fisher[6] and Schwimmer[7] which have been used in all of the experiments described in a series of the author's report.

Taka α-amylase : Crystalline sample prepared by the method of Akabori[8] was kindly furnished by Mr. Nishitai of Hyogo Agricultural College.

Salivary α-amylase : This was prepared in high purity from human saliva by Meyer's[9] method.

Sweet potato β-amylase : This was prepared in the grade of purified paste by Ball's[10] method.

Soy bean β-amylase : This was prepared from aqueous extract of soy bean by Fukumoto's[11] method.

These various amylase solutions were treated with Osvan either at 50° for 10 min. or at pH 9.0, 30°C for 20 min. The details of the treatment were described above.

The activities remained were determined. They are shown in Table 9.

Table 9. Inhibitory effects of Osvan treatments on the various amylases.

Amylase	50°C 10min.		pH 9.0, 30°, 20min.	
	*RA (%)	Inhibition(%)	*RA (%)	Inhibition (%)
Malt-α	100	0	100	0
Taka-α	100	0	100	0
Salivary-α	100	0	100	0
Malt-β	0	100	0	100
Soy bean-β	60	40	10	90
Sweet potato-β	30	70	3	97

*RA : Remaining activity.

The stability of various amylase for the Osvan inhibition were different. The α-amylases obtained from any one of the various sources were so stable that no loss of activity was found irrespective of the method. The β-amylase was labile against Osvan treatment. At present, it is difficult to give a satisfactory explanation to such a differrence in the inhibitory effect. It might be due to the special property of enzyme or the presence of the impurities in the samples.

V. SEVERAL INVESTIGATIONS ON OSVAN METHODS

The two enzymes, i. e., α- and β-amylase, in the malt extract can be separated by partial inactivation or by adsorption technique as described in the introduction of this paper. The best method for the separation of the α-amylase from the accompanying β-amylase seems to be the one developed by Ohlsson. Its modifications have been published by several authors.[12] As pointed out by some of the authors, however, the differences of these behaviors, stability against heating and acidity for adsorbents, and protection by Ca-ions against heating are considerably obscure. The Osvan methods developed by the author can be used at any concentrations of malt extract and pure amylases.

In an attempt to investigate the mechanism of the Osvan inhibition on β-amylase, the following experiments were carried out.

1. Absorption Spectra. The ultraviolet absorption spectram were measured with amylase solutions using "Shimadzu" photoelectric spectrophotometer.

(A) The treatment with Osvan-$CaCl_2$ solution at 50° for 10min. 5ml. of α- and β-amylase solution were mixed with 5ml of Osvan $CaCl_2$ solution and heated at 50° for 10min. (Curve 1, 2 in Fig. 7A). As blank tests, the amount of amylase solution were replaced with water (Curve 3), amylase solutions were mixed with water instead of mixing with Osvan-$CaCl_2$ solution (Curves 4, 5). The absorption spectra were shown in Fig. 7A.

(B) The treatment with Osvan-$CaCl_2$ solution at pH 9.0 for 20 min. 5 ml of pure α- and β-amylase solution were mixed respectively with 0.1% Osvan and 0.1 *M* $CaCl_2$ in final. After their pH were adjusted respectively to 9.0 with N/40

NaOH, they were diluted with water to 10ml. in total volume. These were allowed to stand at 30° for 20 min., and then, their pH were reduced to 5.0 by adding 0.1 *M* acetate buffer. Immediately after the addition of the acetate buffer their absorption spectra were measured. (Fig. 7B).

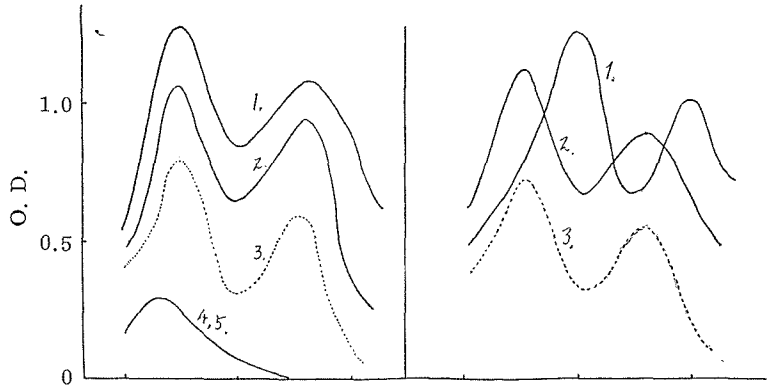

Fig. 7A Fig. 7B

Fig. 7. Ultraviolet absorption spectra of amylases treated with Osvan. 1, β-amylase treated ; 2, α-amylase treated ; 3, Osvan ; 4, α-amylase untreated ; 5, β-amylase untreated.

As can be seen in Fig. 7A, the β-amylase treated with Osvan shows a higher optical density than that of the α-amylase treated under the same condition, while, both amylases untreated with Osvan show no difference in the shape of the spectrum. In Fig. 7B, the position of first peak of the spectrum with the β-amylase treated slightly shifts to the right side compared with the first peaks of Osvan and α-amylase treated. The difference in the shape of the spectrum between the amylases treated and not treated seems to suggest the presence of different complexes of amylases with Osvan.

2. Amount of Osvan Combined with Amylase. It is conceivable that the difference of amount of Osvan combined with amylase cause different Osvan inhibition on amylases. To determine the amount of combined Osvan, the experiment was carried out by dialysis technique, which was used by Klotz[13] (1946) in his experiment of combination of methylorange with albumin. α- and β-amylase were adjusted to about the same activity which were compared with each other by the reducing power and to the same quantity of protein. 5ml. of amylase solution was mixed with equal volume of 0.2 % Osvan solution containing 0.2 *M* $CaCl_2$ and the mixture was then treated at 50°, for 10min, or at alkaline pH 9.0 as mentioned above. In the former case the sample was diluted with 5 ml. of distilled water, and in the latter case it was neutralized with 5ml. of acetate buffer, and the sample was kept in the cellophan bag to dialyse against distilled water 50ml. The dialysis was carried out until equilibrium state, for about 3 days After the dialysis, the amount of free Osvan in external solution was measured photometrically at 530 mμ, by using eosin as indicator. The standard curve of free Osvan was used for the calculation.

Table 10. Comparison of the amount of Osvan combined with amylase.

Osvan-treatment	Sample (amylase)	Contents of sample		Amount of Osvan combined (γ)	$CaCl_2$**
		Protein γ/ml	E.A*		
at 50° for 10min.	α_1	44	100	66	−
	α_2	44	100	52	+
	β_1	45	95	122	−
	β_2	45	95	168	+
at pH 9.0	$\alpha^{(I)}$	44	100	57	+
	$\beta^{(I)}$	45	95	146	+

* Enzyme activity ; this was shown by a arbitrary unit.
** The signs(+) and(−) indicate on the Osvan treatment with or without $CaCl_2$.

Table 10 indicates that the affinity of Osvan to β-amylase is larger than of the α-amylase. On the treatment at 50° for 10 min., $\alpha_2 : \beta_2 = 52 : 168$ and $\alpha(I) : \beta(I) = 57 : 146$, namely, amount of Osvan combined with β-amylase was about 3 times of that of Osvan combined with α-amylase. Ca-ion effected to reduce the combination of Osvan against α-amylase and stimulate it against β-amylase, e. g. $\alpha_1 : \alpha_2 = 66 : 52$, but $\beta_1 : \beta_2 = 122 : 168$. Such effects will support general conception that the additon of Ca-ion stabilize α-amylase greatly and inactivate β-amylase. Such remarkable difference of combining power of Osvan against α- and β-amylase may be a factor of Osvan inhibition solely on β-amylase.

3. The Effect of Proteolysis. It is considered that native protein is not digested by enzyme but denatured one is digested easily, and the degree of denaturation of proteins is estimated by proteolysis. Okunuki[14] measured the degree of denaturation of Taka-α-amylase (amylase of Asp. *oryzae*) by using proteolysis and reading an optical density at 278 mμ. The author examined the degree of denaturation of the malt amylase tested by the Okunuki's method

(A) Proteolysis on the case of Osvan-$CaCl_2$ treatment at 50°C for 10min. : 2ml of amylase solution was mixed with equal volume of 0.2% Osvan containing 0.2 *M* $CaCl_2$, and heated at 50° for 10min. After treatment the 1 ml. of 0.1% papain solution was added, and incubated at 30° for 60min. 1 ml. of 30% trichloroacetic acid (T. C. A.) was added to the hydrolysate. Supernatant was separated from the precipitate and the optical density was measured at 278 mμ.

Since these values must be corrected by the blank test, the mixture of amylase and Osvan-$CaCl_2$ was not heated, and then treated as above. The corrected value of O. D. was calculated by reduction of the blank reading from the reading of treated sample. Completely denatured enzyme solution was obtained by heating at pH 12, 50° for 10min., until the complete loss of activity. This sample was proteolyzed and measured photometrically and calculated as corrected value as above.

The ratio of denaturation, abbreviated as RD, of the test solution was calculated from the following equation :

$$RD(\%)=\frac{\text{OD test}-\text{OD blank}}{\text{OD den.}-\text{OD blank}}\times 100$$

where, OD test, OD blank and OD den. denote the optical density (at 278$m\mu$) of the supernatant of the digested solution, of the test, blank (native) and completely denatured enzyme, respectively.

(B) Proteolysis in the case of Osvan Ca-treatment at pH 9.0 : The mixture of 2ml. of amylase solution, 2ml. of 0.2% Osvan solution (containing 0.2M $CaCl_2$) and 1 ml. of N/40 NaOH was heated at 30° for 20 min., and was neutralized with 1 ml. of acetate buffer (pH 4.7), and then proteolysis, precipitation with TCA, and photometric reading were carried out as above.

Table 11. "Rate of Denaturation" of Osvan-treated amylases measured by proteolysis.

Osvan-treatment at	Amylases	Reading at 278 mμ			R.D(%)	Rate of inactivation.(%)
		OD test	OD blank	OD den.		
50°, 10min.	α-	0.293	0.292	0.328	2.6	0
	β-	0.286	0.247	0.288	95.1	100
pH 9.0	α-	0.449	0.446	0.491	6.6	0
	β-	0.405	0.361	0.405	100.0	100

* This was measured by activity remained.

From the result of Table 11, it was ascertained that denaturation of β-amylase by Osvan treatment was almost complete but that of α-amylase was a few and the rate of inactivation of amylase was related to the degree of denaturation of enzyme protein. The experiment shows that the inactivation of β-amylase by Osvan is characteristic.

The results of investigation on the effect of a series of Osvan-Ca treatments on malt amylases have all led to the conception of the characteristic denaturation and inactivation of β-amylase. It seems likely that the Osvan α-maylase complex retains its initial amylase activity, whereas the Osvan-β-amylase-complex loses its activity easily.

It is very interesting and difficult to interpret the mechanism of combination of amylase with detergent A probable explanation of the difference in inhibiting effect is that in the case of Osvan, in addition to simple ionic binding, there is possibility of association of the long hydrocarbon chains with peptide chains of the enzyme protein. This is known by Putman[15] (1948) in the case of SDS. It may be inferred that inhibition with Osvan on amylases is influenced not only by the number of acidic groups of enzyme protein, but also its distribution, net charge of protein and other conditions.

Explanation about the differential behavior of Ca-ion on two amylases is not yet known though from the experimental results the author considers that Ca-ion will be a protector to stabilize the formula of α-amlase and it does not combine with peculiar site of enzyme.

According to Gorini[16], a protective effect of Ca-ion on inhibition of trypsin

at alkaline site will be as follows: Ta (active)⇄Ti (reversible inactivation). In this case, Ca-ion makes the reaction reverse (Ta←).

VI. THE EFFECT OF DETERGENTS ON MOULD AMYLASE

A lot of studies on mould amylase have been made in Japan. In the present paper, the author described on the examination of a technique to separate component each from a mixture of Taka α-amylase (amylase of Asp. *oryzae*) and malt α-amylase. According to Ran[17] (1948), by treatment at pH 1.8 malt α-amylase was inhibited immediately, though mould amylase decrease its activity gradually and irreversibly. This technique, however, is not favorable, for mould α-amylase loses the activity more or less.

The author used anionic- (SDS) and cationic-(Osvan) detergent to separate each component from the mixture of two kinds of α-amylase, which were prepared from malt extract and SANKYO-Taka diastase as described previously.

1. Separation of Taka-α-Amylase from Accompanying Malt α-Amylase. Effect of pH on the α-amylase inhibition by SDS treatment (0.025% in final, at 30°C for 15min.) is shown in Fig. 8.

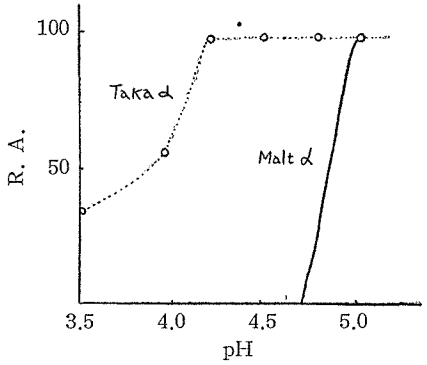

Fig. 8. Effect of pH on SDS treatment (in final concentration 0.025 %).

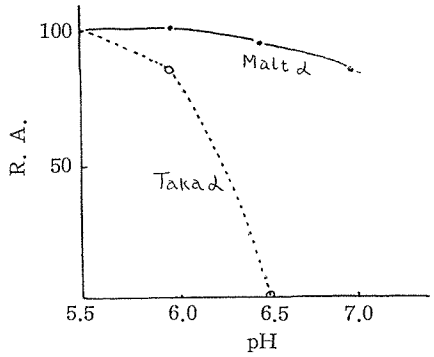

Fig. 9. Effect of pH on Osvan treatment (in final concentration 0.04 %).

In Fig. 8, activity of Malt-α-amylase fell down to zero at pH 4.7, whereas Taka-α-amylase was completely stable at pH 4.2 to 4.7. From the result, following method of separation would be pointed out, i.e. treatment of the mixture of amylases with SDS 0.25 % in final at 30°C, pH 4.5, for 15 min. Using this method it was observed that malt-α-amylase was inhibited completely, while Taka-α-amylase kept its initial activity.

2. Separation of Malt-α-Amylase from Accompanying Taka-α-Amylase. Effect of pH on the α-amylase inhibition by Osvan treatment, i.e. Osvan 0.04 % in final at 40° for 15 min., was shown in Fig. 9.

As can be seen in Fig. 9, Taka-α-amylase was very feeble at alkaline pHs. In the case of pH 6.5, Taka-α-amylase lost its activity completely, whereas malt-α-amylase lost only 10 %. Thus this can not be used for quantitative separation of malt-α-amylase, though it is available for general separation.

A probable explanation for different inhibition on both α-amylases is that

the isoelectric points of both amylases stand far apart from each other, i.e. pH 5.7-6.0 for malt α-, and pH 3.7-4.2 for Taka-α-amylase, and the inhibition by SDS or Osvan is severe on the acid or alkaline side of isoelectric point of amylase respectively.

Therefore, pH 4.5 adopted for the SDS treatment would be inhibitory against malt-α-amylase, and pH 6.5 for the Osvan treatment would be inhibitory against Taka-α-amylase.

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